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AUTHOR(S): LESI C; D'ERIL G V M; ZONI L; MALAGUTI P
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Conformational Changes in Chymotrypsin* Detected with Antibodies, II[1]

Antibody-interactions with chemically modified and denatured chymotrypsins

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Summary: The resistance of chymotrypsin, DIP-chymotrypsin and chymotrypsinogen to urea-denaturation was tested immunologically. Chymotrypsin is quickly denatured by urea, while DIP-chymotrypsin and chymotrypsinogen show prolonged retention of their antibody-combining ability. Conformational changes are held responsible for the increased stability of DIP-chymotrypsin. Carboxymethylation of chymotrypsin, but not of chymotrypsinogen, abolishes immune-precipitation, while one antigenic determinant is retained. A conformational change of CM-chymotrypsin on inactivation with DFP gives rise to a second antigenic determinant and restores precipitability. Guanidation of ε-NH_2 groups yields a precipitable product, in contrast to carboxymethylation. Basic groups are integral parts of most antigenic determinants of chymotrypsin. Their spatial requirements for antibody fixation are different.

Zusammenfassung: *Nachweis von Konformationsänderungen in Chymotrypsin durch Antikörper, II: Antikörperreaktionen mit chemisch modifizierten und denaturierten Chymotrypsinen.* Die Resistenz von Chymotrypsin, DIP-Chymotrypsin und Chymotrypsinogen gegen Harnstoffdenaturierung wurde mit immunologischen Methoden untersucht. Chymotrypsin wird schnell durch Harnstoff denaturiert, während DIP-Chymotrypsin und Chymotrypsinogen die Fähigkeit, Antikörper zu binden, lange beibehalten. Die erhöhte Stabilität von DIP-Chymotrypsin wird auf eine Änderung der Konformation zurückgeführt.
Carboxymethylierung von Chymotrypsin — nicht von Chymotrypsinogen — führt zu einem Verlust der Immunfällung, jedoch bleibt eine antigene Determinante erhalten. Eine Konformationsänderung des CM-Chymotrypsins durch Inaktivierung mit DFP führt zur Bildung einer zweiten Determinanten und damit zur Wiederherstellung der Fällbarkeit.
Guanidierung der ε-ständigen NH_2-Gruppen ergibt im Gegensatz zur Carboxymethylierung ein fällbares Produkt. Basische Gruppen sind wesentliche Bestandteile der meisten antigenen Determinanten vom Chymotrypsin. Ihre räumlichen Erfordernisse für die Antikörper-Fixierung sind verschieden.

Abbreviations:
DFP = diisopropylfluorophosphate; DIP-chymotrypsin = diisopropylphosphoryl-chymotrypsin; DC-chymotrypsin = diphenylcarbamoylchymotrypsin; CM- = carboxymethyl-.

* Chymotrypsin A (EC 3.4.4.5).

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[1] 1. Commun.: G. GUNDLACH, diese Z. 351, 690 [1970], preceding.

Antigen-antibody reactions are surface phenomena in which the two reactants approach each other to a minimal distance of 1 Å [2]. The combining site of the antibody has dimensions of $25-30 \times 10 \times 6$ Å [3]. Thus, in small antigens, a sizable area of their surface is covered on antibody attachment. Chymotrypsin belongs to this group of antigens. Its dimensions are $45 \times 35 \times 38$ Å in the crystalline state[4] and neither size nor conformation should be significantly different in solution[5].

The enormous specificity of antigen-antibody reactions was first described by LANDSTEINER[6], some 40 years ago. Antibodies are able to discriminate between protein-bound *p*-aminobenzoic acid and *o*-aminobenzoic acid, *p*-arsanilic acid or *p*-toluidine[7]. It was therefore not surprising that DIP-chymotrypsin, chymotrypsin and chymotrypsinogen could be quantitatively differentiated by immunological means[1]. These differences as well as the enhanced immune reaction of chymotrypsin by indole were attributed to varying conformations of the antigens. Previous investigations of conformational changes of chymotrypsin on inhibitor binding, inactivation with DFP and during the transition from chymotrypsinogen to the active enzyme with different techniques gave less clear results. While x-ray data indicated major conformational changes of carboxypeptidase upon substrate-binding[8], a similar effect of inhibitors on lysozyme and chymotrypsin was not observed with certainty[9]. SIGLER and SKINNER[10] found γ-chymotrypsin and its DIP-derivative crystallographically indistinguishable, having very similar diffraction patterns and concluded that only a minor if any conformational difference exists. Although optical rotatory dispersion measurements revealed structural changes during zymogen-activation[11], the helical content of chymotrypsinogen and chymotrypsin appeared to be identical[4,12].

In this paper, the usefulness of immunological methods for the determination of conformational changes is further substantiated by studying the course and extent of antigen-denaturation by urea and the influence of chemical modification on the antigenic properties of chymotrypsin.

Experimental

Materials

All *chemicals* used were of analytical grade or the best quality commercially available unless otherwise stated.

Bovine chymotrypsinogen, *chymotrypsin* and *DIP-chymotrypsin* were prepared as previously described[1].

DC-chymotrypsin was obtained in analogy to DIP-chymotrypsin by the reaction of chymotrypsin with diphenylcarbamoylchloride.

CM-chymotrypsin: A mixture of 200 mg chymotrypsin dissolved in 2.5 ml H_2O and 2.5 ml of a neutralized solution of 135 mg iodoacetic acid was kept for 6 h at 25°C in a pH-stat with continuous addition of NaOH to maintain a pH of 10. The solution was then acidified to pH 5.0, dialyzed against running distilled water and lyophylized. Amino acid analysis of the product revealed 6.3 mol of dicarboxymethyllysine, 1.6 mol of monocarboxymethyllysine and 4 mol of unreacted lysine per mol of chymotrypsin. 30% of the enzymatic activity was lost by carboxymethylation (chloroacetyl-L-tyrosine methylester as substrate).

DIP-CM-chymotrypsin was prepared by DFP-treatment of CM-chymotrypsin. The product had no enzymatic activity.

Guanidated chymotrypsin was prepared according to[13] by the reaction of *O*-methylisourea with chymotrypsin at 1–2°C and pH 10.4. The dialyzed and lyophylized product contained 12.5 mol of homoarginine and 1 mol of unreacted lysine and retained 70% of the

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enzymatic activity of native chymotrypsin with chloroacetyl-L-tyrosine methylester as substrate.

Antisera against DIP-chymotrypsin were obtained from rabbits as previously described[1].

Methods

Qualitative immune reactions: Geldiffusion[14] and immunoelectrophoretic[15] procedures were used.

Quantitative determination of immune-precipitates: Mixtures of 2—20 µg antigen in 0.05 m*l* 0.1M barbital buffer pH 8.2 were incubated with 0.05 m*l* antiserum for 1 h at 38°C, then kept for 2 h at 4°C. The precipitates were collected by centrifugation, washed with saline and dissolved in sodium carbonate solution for protein assay by the LOWRY method[16].

Determination of the equivalence point of antisera: 0.05 m*l* aliquots of antiserum were incubated for 1 h at 37°C with 1—20 µg antigen in 0.02 m*l* 0.1M barbital buffer pH 8.2 and the resulting precipitates removed by centrifugation. 0.002 m*l* of each supernatant was pipetted into a hole of 1.5 mm diameter in an agar-slab on a 76×26 mm slide (2.5 m*l* 1% Reinagar Behringwerke in 0.1M barbital buffer pH 8.2). After the holes were emptied by diffusion, they were refilled with 0.002 m*l* antiserum to detect excess antigen in the supernatants. The last completely precipitated antigen-concentration was considered to be the equivalence point of the antiserum tested.

Antibody-adsorption test: Urea-treated antigens were incubated with anti-DIP-chymotrypsin and their antibody-binding ability tested in the following manner:

Two parallel grooves were cut 1 cm apart into an agar-slab on a 20×76 mm slide (s. a.) and holes punched out at half-distance. The upper groove was filled with fresh antiserum, the lower with antiserum previously exposed to variously treated antigens. The holes were then filled with native (right) or treated antigen (left). Fig. 1 shows the three types of reactions possible: a) The antigen is resistant to the treatment and can therefore exhaust the antiserum. b) The antigen has lost part of its antibody-binding capacity and does not absorb all the antibodies from the serum. c) All the binding sites of the antigen have been destroyed by the treatment and the antibody-titre of the serum is unchanged by addition of the denatured antigen.

The test accounts only for irreversible structural changes and not for possible renaturations after dilution of the 8M urea solution.

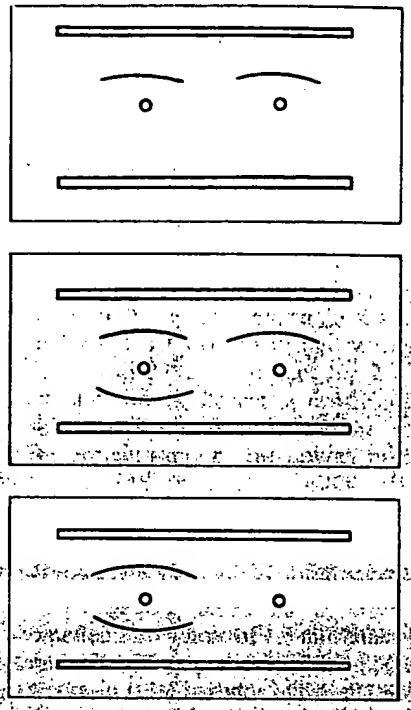

Fig. 1. Antibody adsorption test. Upper groove: fresh antiserum; lower groove: antiserum exhausted with antigen to be tested. Right hole: treated antigen; left hole: native DIP-chymotrypsin.
a) = antigen resistant to treatment.
b) = antigen partially resistant to treatment.
c) = antigen not resistant to treatment.

Results and Discussion

Immune reactions of urea-denatured DIP-chymotrypsin, chymotrypsinogen and chymotrypsin

NEURATH, *et al.*[17] observed an increased viscosity and a change of the optical rotatory dispersion of chymotrypsin and chymotrypsinogen after treatment with urea. Chymotrypsin was found to be more sensitive to urea-denaturation than chymotrypsinogen or DIP-chymotrypsin[18,19]. These findings can be supported by immunochemical experiments. 1% solutions of DIP-chymotrypsin, chymotrypsinogen or chymotrypsin were incubated in phosphate-buffered 8M urea at pH 6.9. The urea-

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reaci on was stopped by a 10-fold dilution with H_2O and . s effect measured by the antibody adsorption test.)IP-chymotrypsin was resistant to urea over a 2 h ncubation-period at 0°C. The treated antigen rema.ned fully precipitable by the antibody (Table 1). I.cubation with urea for 1 h at 30°C still had no effect, but pronounced denaturation was detected after 3 h at 30°C; the antigen had lost part of its precipitating capacity and a reaction pattern as in Fig. 1b was obtained. Incubation for 2 h at 40°C finally resulted in complete denaturation (Fig. 1c).

Table 1. Precipitation of DIP-chymotrypsin treated with urea at 0°C and pH 6,9. Immune-precipitation by addition of 0.05 m*l* antiserum to 6 µg antigen in 0.02 m*l* solution + 0.02 m*l* buffer.

Duration of treatment [min]	Amount of precipitate "Lowry-color" (E_{750})
0	0.635
5	0.665
10	0.670
15	0.658
30	0.650
60	0.665
120	0.660

Chymotrypsinogen showed, like DIP-chymotrypsin, a strong resistance towards urea-treatment and was not denatured after 1 h at 30°C (Fig. 1a). In contrast to its zymogen and DIP-derative, the native enzyme chymotrypsin was found to be extremely sensitive to urea. An incubation of only 1.5 min in 8M urea at 30°C denatured the enzyme completely (Fig. 1c) and even after 0.25 min at 30°C, a partial denaturation was already visible (Fig. 1b). Incubation experiments at different temperatures showed that 10 min at 0°C was tolerated by chymotrypsin (Fig. 1a), 10 min at 10°C denatured the enzyme in part (Fig. 1b) and it was fully denatured after 10 min at 20°C (Fig. 1c). It is evident from these results, that the introduction of the diisopropylresidue and a concomitant conformational change renders DIP-chymotrypsin less susceptible to urea-denaturation than the active enzyme. This increase in stability can also be concluded from our previous diazotation experiments with DIP-chymotrypsin in 8M urea at 0°C. Compared to chymotrypsin, one histidine and one tyrosine residue remained uncoupled, obviously because they were inaccessible to the diazobenzene sulfonate[20] reagent.

Immune reactions of chemically modified chymotrypsins

The introduction of a diphenylcarbamoylresidue at the catalytic site of chymotrypsin yielded a product with the same antigenic properties as DIP-chymotrypsin (Fig. 2). DC-chymotrypsin and DIP-chymotrypsin resemble in this respect ribonuclease A and B[21].

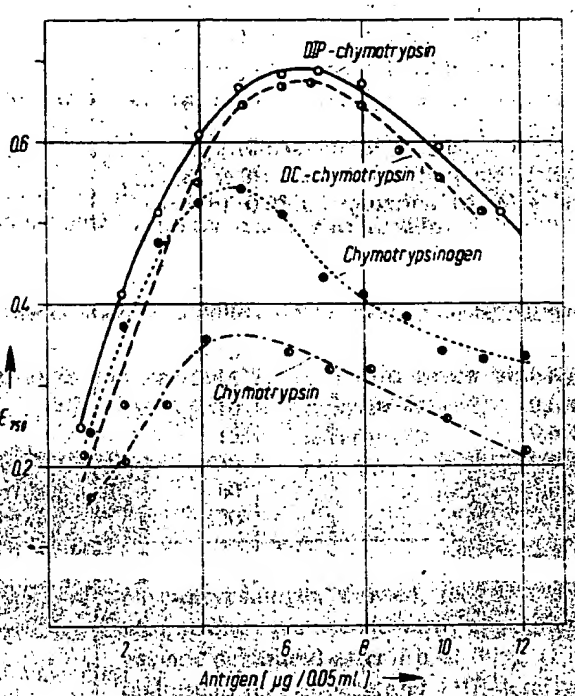

Fig. 2. Comparison of the antigenic properties of DIP-chymotrypsin and DC-chymotrypsin. Assay of immune-precipitates obtained with increasing amounts of antigen (abscissa) with a constant concentration of antiserum. Ordinate: "Lowry-color" at 750 nm.

Alteration of aminoacid sidechains by the introduction of anionic and cationic functions may not necessarily change the conformation of chymotrypsin but can change its immunological reactivity as a consequence of the modified ionic character. Most interesting in this respect is the carboxymethylation of chymotrypsin by iodoacetic acid. Two thirds of all lysine-residues were substituted to give ε-NH_2 mono- and dicarboxymethylderivatives. The product was not able to form an immune precipitate. In contrast to CM-chymo-

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trypsinogen closer analysis showed, that in spite of the lack of precipitation, one antibody-binding site was retained. Preincubation with CM-chymotrypsin rem ved about 44 % of the antibody present in anti-DIP-chymotrypsin serum, resulting in a shift of the equivalence point of the serum from 9 µg to 5 µg DIP-chymotrypsin/0.05 m*l* (Fig. 3).

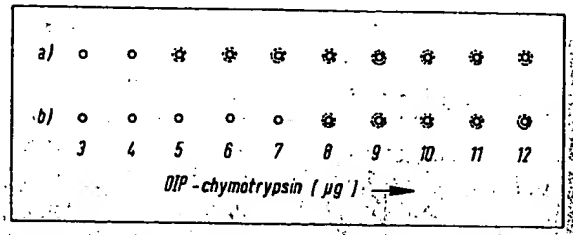

Fig. 3. Determination of the equivalence point by back-titration of antisera. a) 0.05 m*l* serum preincubated with 20 µg CM-chymotrypsin in 0.02 m*l* and then with increasing amounts of DIP-chymotrypsin, b) in 0.05 m*l* serum incubated only with DIP-chymotrypsin.

CM-chymotrypsin has 70 % of the enzymatic activity of chymotrypsin.

The enzymatic activity is completely lost after treatment with diisopropylfluorophosphate. This inactivation has an effect on the antigenic properties. CM-DIP-chymotrypsin did not only bind antibody, like CM-chymotrypsin but gave a precipitate. The conformational change brought about by the inactivation must thus raise the number of antigenic determinants, which are required to achieve precipitation, to a minimum of two. That the DIP-residue itself does not supply this additional determinant, has been shown previously[1]. Moreover, it can be assumed from experiments with ribonuclease, that this is also true for the catalytic site as such. Carboxymethylation of one histidine residue (His-12 or His-119) at the active site of ribonuclease does not alter the antigen-antibody binding curve[22]. The difference between the immune reactions of CM-chymotrypsin and CM-DIP-chymotrypsin resembles to a lesser extent the enhanced antibody fixation of chymotrypsin by indole[1]. An interesting but different approach to characterizing single antigenic determinants as in CM-chymotrypsins is the isolation of an antibody against the *C*-terminal heptapeptide of myoglobin[23] from a mixture of antibodies[24]. The isolated antibody binds myoglobin but does not precipitate it.

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The question of whether the ε-NH_2 groups of lysine sidechains are integral parts of antige ic determinants was further investigated by ba ic substitution. Guanido-chymotrypsin remained p: cipitable by antibodies. Since the degree of gua dation was even higher than that of carboxymethy ation and since the spatial requirements of guanid and carboxymethylgroups are of the same order o magnitude, basic functions seem to be importan constituents of the antigenic determinants o chymotrypsin. At least one antigenic determinan is lost by guanidation. Antiserum exhausted with guanido-chymotrypsin still precipitated additional DIP-chymotrypsin (Fig. 4). This may be explained by a different degree of participation of the ε-NH_2 groups of various antigenic determinants in the fixation of antibody or by the induction of a limited conformational change on guanidation, resulting in the loss of one antigenic determinant. The reactivity of chemically modified chymotrypsin *versus* anti-DIP-chymotrypsin is summarized in Table 2.

Fig. 4. Reaction of guanidated chymotrypsin with antiserum against DIP-chymotrypsin. Upper groove: fresh antiserum; lower groove: antiserum preincubated with guanidated chymotrypsin. Left hole: DIP-chymotrypsin; right hole: guanidated chymotrypsin.

Table 2. Reaction of modified chymotrypsins with antiserum against DIP-chymotrypsin.

Substance:	Reaction with antibody
DIP-chymotrypsin	+ + +
DC-chymotrypsin	+ + +
Chymotrypsinogen	+ + +
Chymotrypsin	+ +
CM-chymotrypsin	—
DIP-CM-chymotrypsin	+
CM-chymotrypsinogen	+
Guanido-chymotrypsin	+

+ = Precipitation; — = No precipitation.

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